Analysis of Hoechst side population (SP) on the NPE Analyzer

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Background and Objectives

In this laboratory workshop we will be isolating mouse bone marrow and labeling it with the cell-permeable DNA binding dye Hoechst 33342. Stem cells (including some puripotent progenitors) express the ABCG2 pump molecule, which pumps Hoechst 33342 out of the cell. This exclusion of Hoechst 33342 by stem cells produces a hypodiploid population (termed the "side population", or SP) that is distinguishable when the cells are analyzed on a flow cytometer equipped with an ultraviolet laser. Hoechst 33342 emits primarily in the blue range (around 450 nm) but also has a weaker red emission component. When these two emission wavelengths are detected and plotted against each other, the "side population" can be easily resolved (Figure 1). This population is highly enriched for stem cells, as previously verified with reconstitution studies in lethally irradiated mice. The Hoechst SP assay has become a critical assay for the identification and isolation of mammalian stem cells.

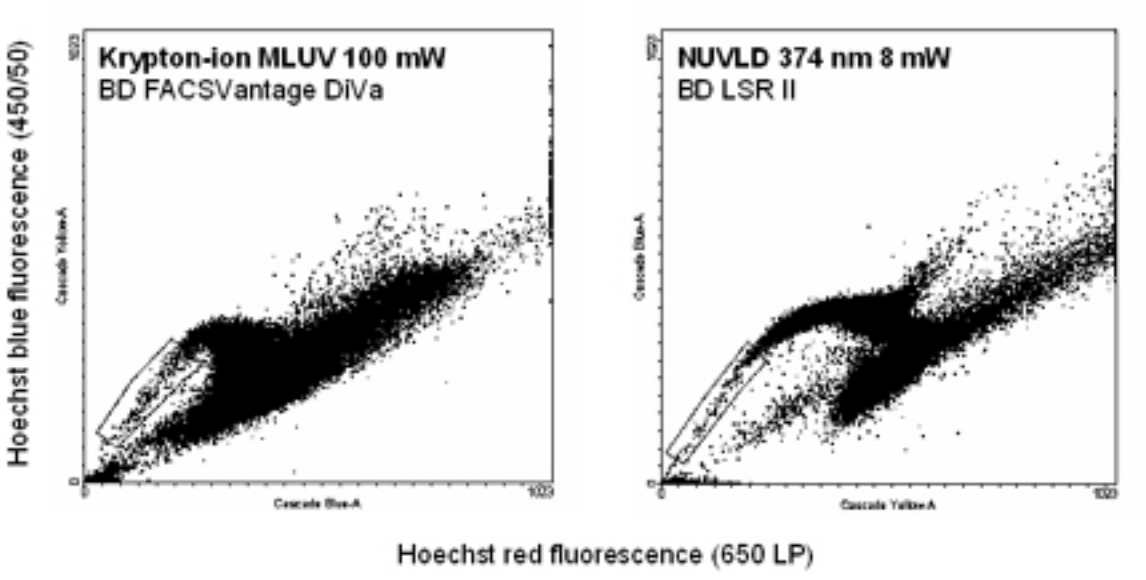

Figure 1. *Hoechst SP analysis of unpurified mouse bone marrow using either a FACSVantage with a UV-emitting krypton-ion gas laser (351 nm, 100 mW, left panel) or the BD LSR II with a near-UV laser diode (374 nm, 8 mW, right panel).*

Hoechst SP analysis has traditionally required a large-scale flow cytometer (such as a BD FACSVantage or Cytomation MoFlo) capable of supporting a water-cooled UV gas laser (such as a krypton-ion source). In our laboratory we have used recent

developments in both instrument and laser technology to establish a lower-cost analysis platform for the analysis of Hoechst SP cells. Our laboratory currently uses one of several recently available solid-state UV lasers for detection of Hoechst SP on smaller benchtop cuvette flow cytometers; **frequency-tripled Nd-YAG lasers** emitting at 355 nm, and particularly **near-UV laser diodes (NUVLDs)** emitting at 375 nm are smaller, cheaper UV sources that give excellent Hoechst SP results. However, the flow cytometers that can accommodate these lasers (such as the BD LSR II) are still relatively complex and expensive.

In response to this problem, we have recently modified the mercury arc-lamp equipped **NPE analyzer** to detect Hoechst SP, equipping it with two PMTs and a dichroic mirror to detect the biphasic Hoechst emission. The standard NPE Analyzer is equipped with a mercury arc lamp, providing an excellent potential source of UV excitation for Hoechst SP analysis. In addition, we have installed a near-UV laser diode on our NPE Analyzer to provide an even stronger source of UV light. In this laboratory we will use both of these instrument configurations to attempt the analysis of Hoechst SP in mouse bone marrow. The NPE Analyzer provides the added benefit of simultaneous electronic volume analysis; we believe that volume measurement may provide a valuable new phenotype for stem cell characterization, distinct from the traditional forward/side scatter measurement.

It should be emphasized that both the analysis of Hoechst SP and volume characterization on the NPE Analyzer is very new; we have as yet generated very little data in this area. This workshop session may therefore generate some very novel and interesting results.

Instrumentation

We will be using an NPE Quanta Analyzer to analyze Hoechst SP (Figure 2).

Figure 2. NPE A*nalyzer.*

This instrument is equipped with two high-sensitivity photomultiplier tubes (PMTs), set at right angles to one another, with a reflecting dichroic holder in the middle (Figure 3a and 3b). A 450/55 nm narrow bandpass filter will be used to detect the Hoechst blue signal, and a 630/22 nm bandpass will be used for the Hoechst red. These filters are have slightly broader bandpass characteristics than those traditionally used for Hoechst SP analysis with higher-power lasers; sensitivity is increased with these filter modifications when using low-power sources such as solid state lasers or the mercury arc lamp.

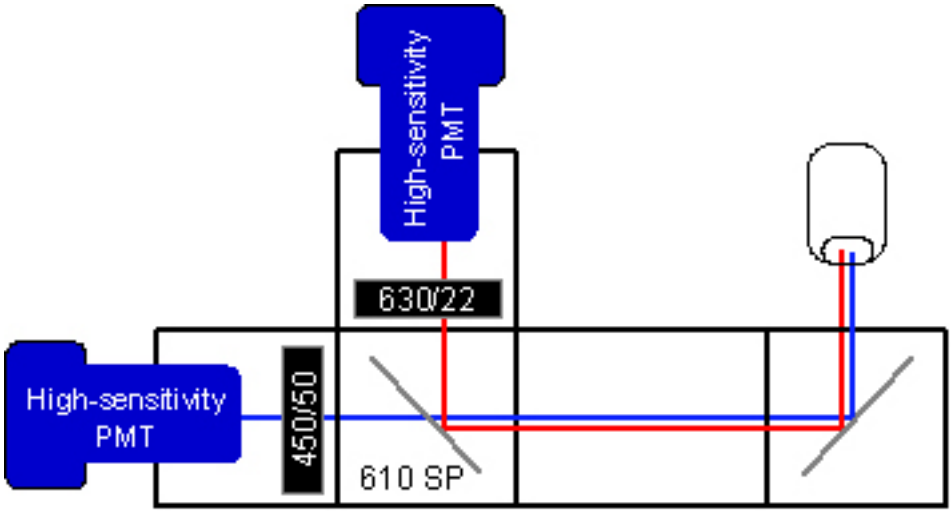

Figure 3a. *Optical layout for the NPE Quanta Analyzer.*

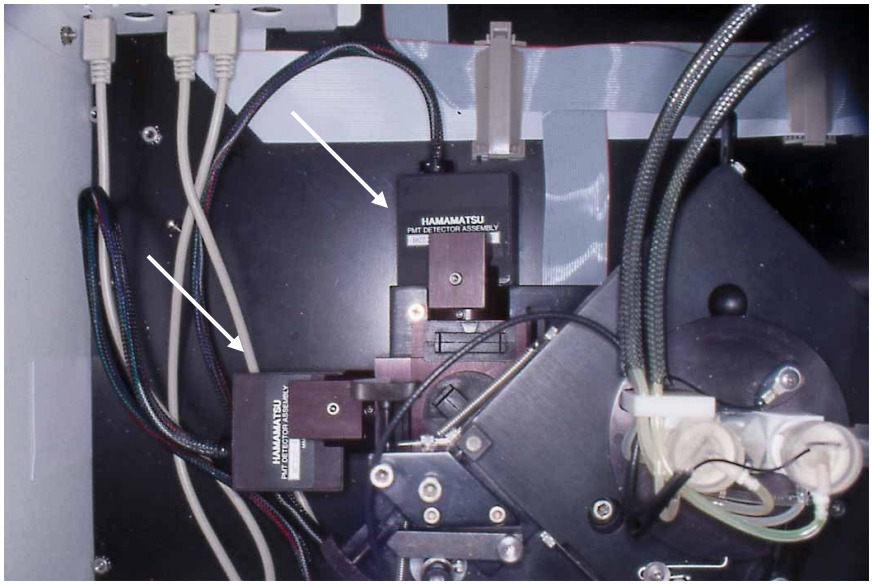

Figure 3b. *PMTs mounted in the NPE Quanta Analyzer*

We will be using two (hopefully) excitation sources; one is the mercury arc lamp traditionally installed on the NPE Analyzer. We use a 120 W lamp with a 365 nm

shortpass filter in front of it to filter the UV lamp line (Figure 4). Despite the high lamp power, however, it is estimated that less than 2 mW of actual UV light actually reaches the flow cell (since the lamp radiates light in all directions. This power level may or may not be sufficient to achieve good Hoechst SP resolution.

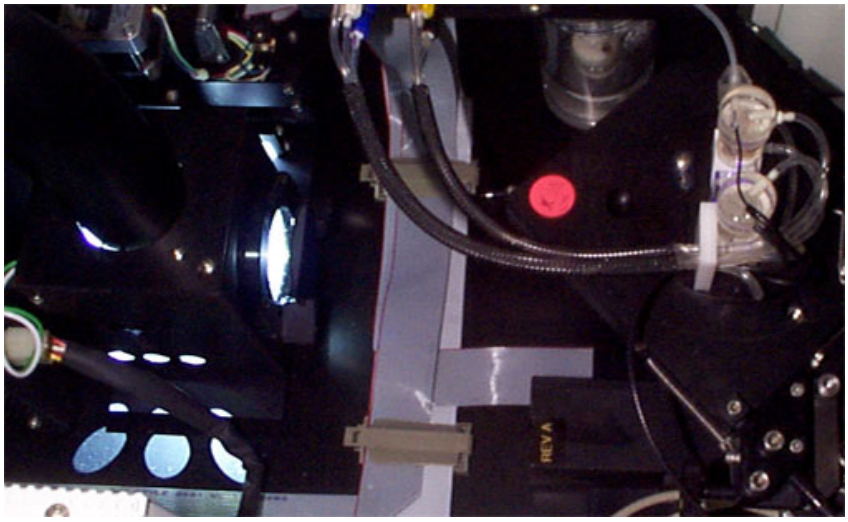

Figure 4. *Mercury arc lamp on the NPE Quanta Analyzer*

We will therefore also be trying a second excitation source, namely a near-UV laser diode (NUVLD) (Figure 5a and 5b). This laser will be mounted in the NPE cabinet and the beam directed to the objective and the flow cell with a steering mirror (Figure 6). The laser we will be using is a Power Technology 374 nm emitting at 8 mW. This laser has given excellent results for Hoechst SP on a BD Bioscience LSR II, suggesting that it should give similar results on the NPE Analyzer.

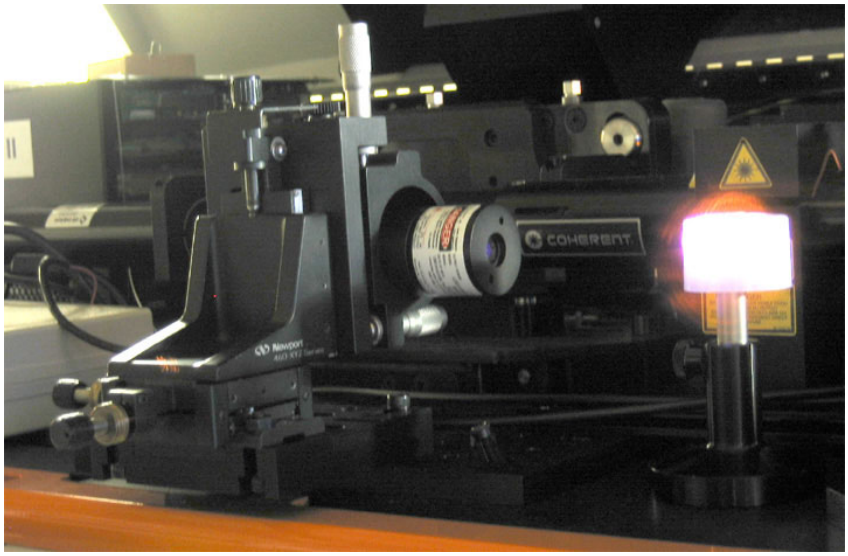

Figure 5a. *Near-UV laser diode (NUVLD) mounted on the BD LSR II.*

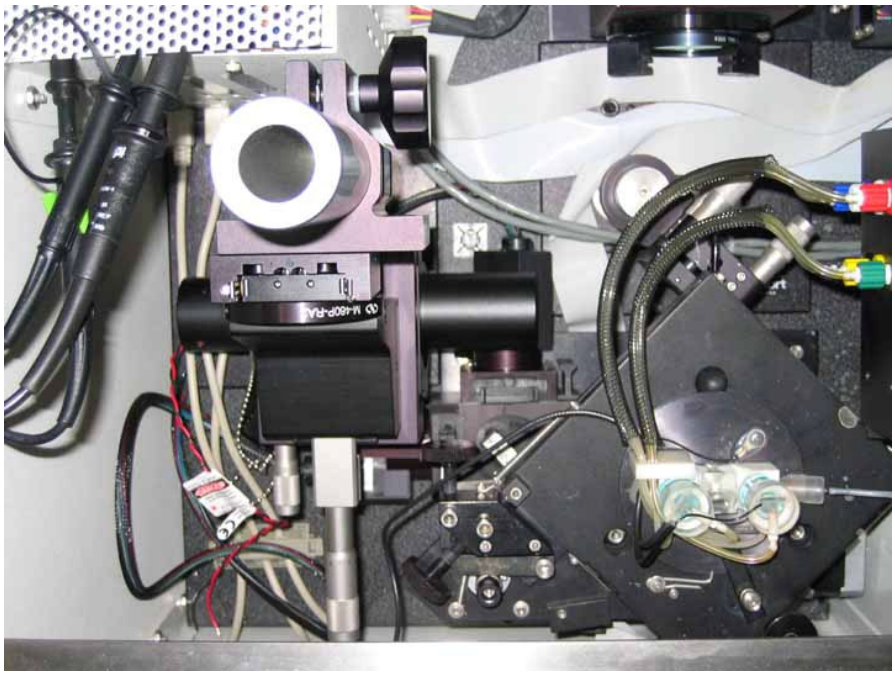

***Figure 5b. Near-UV laser diode (NUVLD) mounted on the NPE Quanta*. Laser mirror is visible beneath the flow cell stage on the right.**

Materials

The following materials will be need for this workshop.

- **Two young mice (any strain).** We will be extracting bone marrow from these animals.

- **A549 cell line**. This lung carcinoma cell line expresses high levels of the ABCG2 pump, and makes a good positive control for the Hoechst SP method.

- **Hoechst 33342.** This is generally available in powder form. A stock solution is prepared at 1 mg/ml in distilled water, and can be stored for up to one month at 4^{o}C in the dark. Concentrated solutions of Hoechst 33342 in phosphate buffers tend to precipitate over time.

- **Hoechst labeling buffer.** Cells are labeled with Hoechst dye in a buffer consisting of Hanks balanced salt solution (HBSS), preferable with no phenol red, containing fetal bovine serum (FBS) at 2% and HEPES buffer at 10 mM. The labeling buffer is a critical parameter for success of Hoechst labeling.

- **Fumetrimorgin C.** This highly specific ABCG2 pump inhibitor completely blocks Hoechst SP activity, serving as a baseline control for SP activity. We usually prepare this drug at a stock concentration of 1 mM in distilled water, and freeze it at -20^{o}C for long term storage. The more general inhibitor **verapamil** may also be used.

- **Nuclei buffer containing Hoechst 33342.** This buffer will be used to prepare Hoechst 33342 labeled trout erythrocytes for instrument alignment. Add 0.5% NP-40 to PBS and allow to enter solution. Then add Hoechst 33342 at 5 µg/ml and store in a foil-wrapped bottle at 4^{o}C for up to one month. Prior to instrument alignment, trout erythrocytes will be added to a small quantity of this buffer and used as an alignment tool on the NPE Analyzer prior to Hoechst SP analysis.

- **UV-excited sensitivity bead array.** We use Molecular Probes InSpeck Blue beads, although arrays from other manufacturers (i.e. Polysciences or Spherotech) can be substituted. These UV-excited bead mixtures consist of an arbitrarily labeled "100%" bead population, and 30, 10, 3, 1, 0.3 and 0% labeled fractions. A "cocktail" of these beads will be used to assess instrument sensitivity prior to Hoechst SP analysis.

- A clinical centrifuge, a waterbath for 37^{o}C incubation, dissection instruments, syringes, hypodermic needles, pipets and sample tubes

Procedure

- Perform humane euthanasia on the mice. Cervical dislocation should be avoided, since it may complicate bone removal with excessive internal bleeding.

- Remove the femurs and tibias from both animals. Attention should be paid to removing as much fat and connective tissue from the bones as possible during the procedure. Bones should be transferred to a dish containing the above labeling buffer.

- Extract bone marrow from each bone using a 10 ml syringe equipped with a 27 gauge needle, filled with labeling buffer. Marrow can be extracted into another buffer-filled dish. Attempt to remove as much bone marrow as possible with repeated washings of the marrow space.

- Once the marrow is completely extracted, change the needle on the syringe to 18 gauge, and slowly draw up the marrow fragments into the syringe; then, slowly expel them into a centrifuge tube. This method should break up the marrow aggregates without damaging the cells.

- Finally, draw up the bone marrow suspension into a wide-bore pipet, and filter through a 100 micron filter into a clean centrifuge tube.

- Centrifuge the cells at 400 x g for 7 minutes at room temperature.

- Resuspend the cells in labeling buffer, filter again through another 100 micron filter into a new centrifuge tube. Remove a small sample for counting by haemocytometer. A young mouse will generally yield between 15 and 30 million nucleated cells from femur/tibia extraction.

- Centrifuge the cells again at 400 x g for 7 minutes at room temperature.

- Resuspend the cells at 5 x 10^6 cells per ml in labeling buffer. Split the bone marrow suspensions from each mouse into two sample of equal volume (one with no drug, one with fumetrimorgin C, four samples total if two mice are used). Place all four tubes in a 37°C waterbath for 10 minutes. If A549 cells are being used as controls, they can be labeled at 1 x 10^6 and can be set up in parallel with the mouse bone marrow samples.

- After 10 minutes, add fumetrimorgin C at 20 µM final concentration to half the samples. If a 10 mM stock is used, for example, dilute at 1:500. Incubate for 30 minutes.

- After 30 minutes, add the Hoechst 33342 to all samples at 5 µg/ml final concentration. If a 1 mg/ml stock is used, for example, dilute at 1:200. Incubate for 90 minutes. Samples should be gently mixed by inversion every 20 minutes or so. During this incubation, cool the centrifuge to 4°C and place a small quantity of labeling buffer on ice.

- After incubation, transfer the tubes immediately to the refrigerated centrifuge and spin at 400 x g for 7 minutes. Resuspend the cells in cold label buffer at 1 x 10^6 per ml and store on ice until analysis. Samples can be kept on ice for up to six hours without significant loss of Hoechst SP population.

Data Acquisition and Analysis

- Turn on the NPE Analyzer and insert the proper filter set (450/50 nm in the FL1 slot, 630/22 nm in the FL2 slot, a 610 SP dichroic in the circular mirror slot).

- **Initial alignment.** Align the instrument through the FL1 blue detector as usual, using NIM DAPI labeled trout erythrocytes. We try to get a trout erythrocyte peak C.V. of less than 1.7%, or a "NPE best value" of greater than 30 when analyzing on a linear scale (Figure 6).

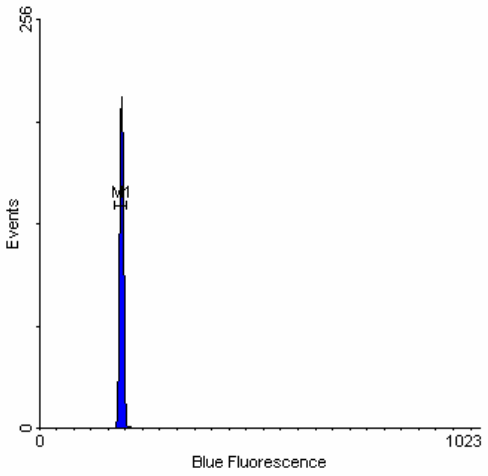

Figure 6. Trout erythrocytes labeled with NIM DAPI reagent.

- **Red channel alignment.** Next, switch to FL2 (Hoechst red fluorescence) as the primary or triggering parameter, also on a linear scale. Prepare trout erythrocytes in Hoechst 33342 nuclei buffer, and run them on the instrument. Realign the instrument through the *FL2 channel* using the manual controls. In addition to the flow cell stage positioning controls, objective focus and lamp X-Y controls, gently rotate the 610 SP dichroic holder to maximize the Hoechst 33342 signal in the FL2 channel. Since Hoechst 33342 has much less fluorescence emission in the red than the blue, we won't see the peak resolution we do in the blue channel; however, we should get a peak C.V. of less than 5% and a "NPE best value" of 20 or more (Figure 7). While aligning on the red, watch the blue as well; the peak C.V. for the blue will degrade somewhat during the red alignment, but it should still remain relatively sharp.

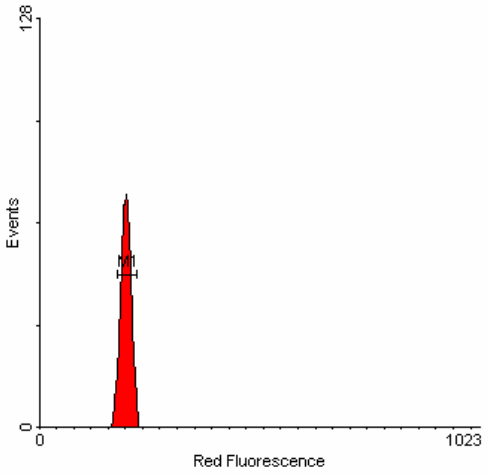

Figure 7. Trout erythrocytes labeled with Hoechst 33342 nuclei buffer and analyzed through the FL2 red fluorescence channel.

- **Sensitivity check on blue channel.** Now, switch to ECV (electronic volume) as the primary or triggering parameter. Run a "cocktail" of Molecular Probes InSpeck Blue beads through the instrument, observing them through the FL1 blue channel set to log scale. We want to be able, at a minimum to distinguish the 100, 30, 10, 3 and 1% beads from the unlabeled population – the 0.3% beads will probably be too dim to resolve (Figure 8). This sensitivity check tells us if the instrument is sensitive enough to resolve the Hoechst SP population. If sensitivity is poor, repeat the above alignment steps.

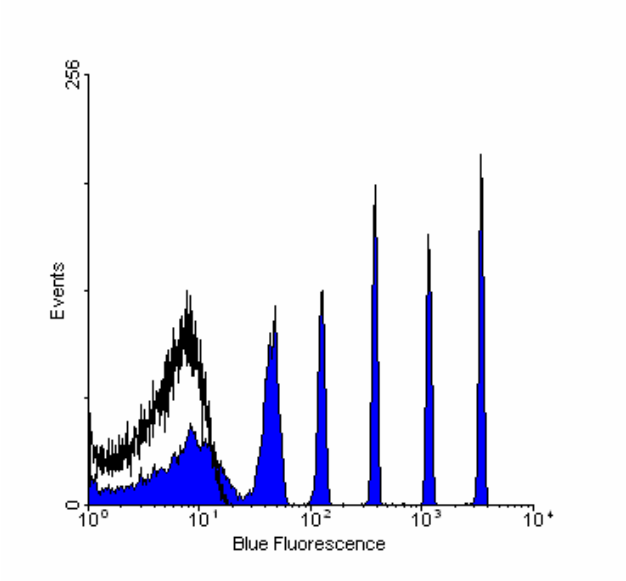

Figure 8. Trout erythrocytes labeled with Hoechst 33342 nuclei buffer and analyzed through the FL2 red fluorescence channel.

- **Hoechst SP analysis.** Finally, run the Hoechst SP samples (with fumetrimorgin C or verapamil controls, and with A549 cell controls if present). Trigger on electronic volume (ECV) and increase the ECV gain until at least two distinct populations are visible. Adjust the FL1 and FL2 detector gains to position the G1 population of the bone marrow at approximately 500 channel units. The Hoechst SP should be visible in the lower left quadrant of the dotplots (Figure 9). nterestingly, the SP appears to b confined to the small cell compartment of the bone marrow based on electronic cell volume measurement (Figure 10).

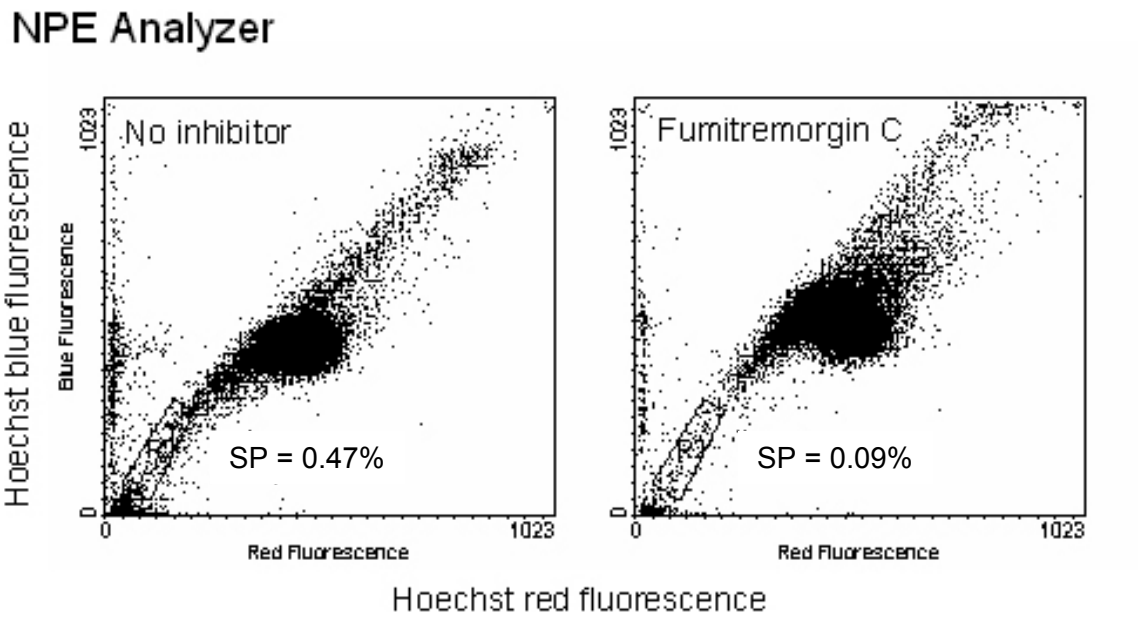

Figure 9. Mouse bone marrow labeled with Hoechst 33342 in the absence or presence of fumimtremorgin C (left and right histograms).

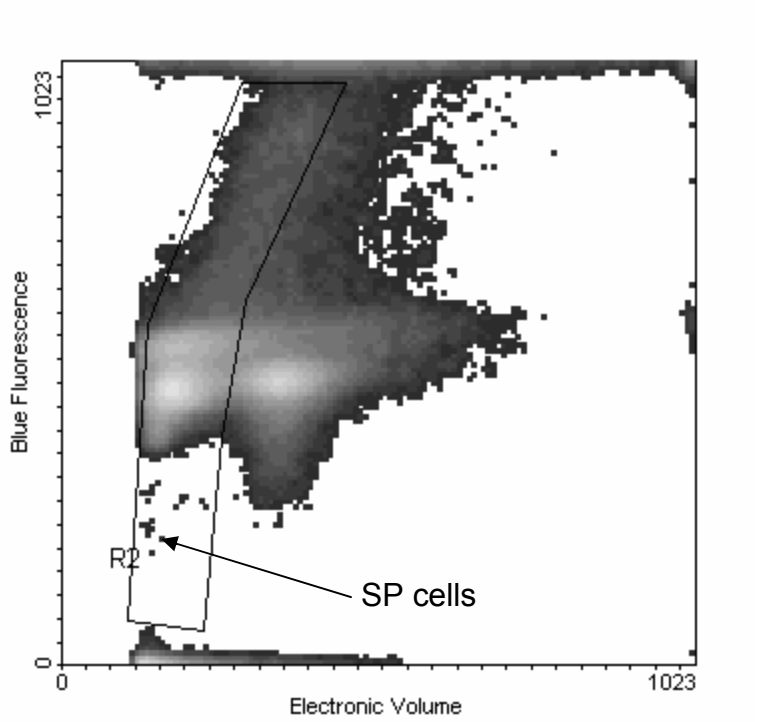

Figure 10. Electronic cell volume phenotype of SP cells.

NUVLD excitation of Hoechst SP cells. Finally, we will (time and materials permitting) install the near-UV laser diode on the NPE and use it as a UV source. Since the power output of the laser at the flow cell should be several times greater than that obtained with the Hg lamp, we expect to see an increase in SP resolution.